FORM PTO-1390 (REV 9-2001) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: GJE-88

U.S. APPLICATION NO. (If known, see 37 CFR 1.5): 10/070568

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/GB00/03460	8 September 2000	8 September 1999

TITLE OF INVENTION: MONOMERIC ANALOGUES OF HUMAN INSULIN

APPLICANT(S) FOR DO/EO/US: You-Min Feng and You-Shang Zhang

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☑ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☐ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☑ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☑ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☐ Amendments to the claims of the International Aplication under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. ☑ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4))_____
10. ☐ An English lanugage translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☑ A FIRST preliminary amendment.
14. ☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 37 CFR 1.821 - 1.825.
18. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. ☑ Other items or information: Certificate of Mailing by Express Mail

U.S. APPLICATION NO. (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
10/070568	PCT/GB00/03460	GJE-88

21. ☑ The following fees are submitted:	CALCULATIONS PTO USE ONLY
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):	
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1040.00**	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890.00**	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$740.00**	
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$710.00**	
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**	
ENTER APPROPRIATE BASIC FEE AMOUNT =	$890.00
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	$
Total claims	17 - 20 =	0	x **$18.00**	$0.00
Independent claims	3 - 3 =	0	x **$84.00**	$0.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$270.00**	$0.00
TOTAL OF ABOVE CALCULATIONS =				$890.00
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.			+	
SUBTOTAL =				$890.00
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				
TOTAL NATIONAL FEE =				$890.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				
TOTAL FEES ENCLOSED =				$890.00
			Amount to be refunded:	$
			charged:	$

a. ☐ A check in the amount of $ _____ to cover the above fees is enclosed.

b. ☑ Please charge my Deposit Account No. 19-0065 in the amount of $ 890.00 to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☑ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 19-0065 . A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

CORRESPONDENCE ADDRESS:

CUSTOMER NUMBER
23,557

David Saliwanchik
SIGNATURE

March 8, 2002
DATE

David R. Saliwanchik
NAME

31,794
REGISTRATION NUMBER

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: U.S. Patent and Trademark Office, Box Sequence, PO Box 2327 Arlington, VA 22202 on December 2, 2002.

David Saliwanchik

David R. Saliwanchik, Patent Attorney

SUBMISSION OF SEQUENCE LISTING
UNDER 37 CFR §§1.821-1.825
Patent Application
Docket No. GJE-88
Serial No. 10/070,568

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	You-Min Feng, You-Shang Zhang
Serial No.	:	10/070,568
Filed	:	March 8, 2002
Conf. No.	:	7193
For	:	Monomeric Analogues of Human Insulin

Box SEQUENCE
Assistant Commissioner for Patents
PO Box 2327
Arlington, VA 22202

SUBMISSION OF SEQUENCE LISTING UNDER 37 CFR §§1.821-1.825

Sir:

Transmitted herewith is a replacement Sequence Listing Under 37 CFR §§1.821 through 1.825 for the above-identified patent application. A Notification of Defective Response Under 35 U.S.C. 371 in the United States Designated/Elected Office (DO/EO/US) was received from the Patent and Trademark Office, and a copy of the Notification is enclosed herewith.

The Sequence Listing is submitted in computer readable format and on paper. I hereby certify that the paper and computer readable copies contain the same information and that no new material is added by this submission.

The Commissioner is hereby authorized to charge any fees under 37 CFR 1.16 or 1.17 as required by this paper to Deposit Account No. 19-0065.

Respectfully submitted,

David Saliwanchik

David R. Saliwanchik
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DRS/la

Attachments: Sequence listing on paper and computer readable format containing the same information; Amendment Under 37 CFR §1.825(a) through (c); copy of Notification of Defective Response.

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Assistant Commissioner for Patents Washington, D.C. 20231 on December 2, 2002

David Saliwanchik

David R. Saliwanchik, Patent Attorney

AMENDMENT UNDER 37 CFR §1.825(a) THROUGH (c)
Patent Application
Docket No. GJE-88
Serial No. 10/070,568

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	You-Min Feng, You-Shang Zhang
Serial No.	:	10/070,568
Filed	:	March 8, 2002
Conf. No.	:	7193
For	:	Monomeric Analogues of Human Insulin

Box PCT/Box SEQUENCE
Assistant Commissioner for Patents
P.O. Box 2327
Arlington, VA 22202

AMENDMENT UNDER 37 CFR §1.825(a) THROUGH (c)

Sir:

In response to the Notification of Defective Response Under 35 U.S.C. 371 in the United States Designated/Elected Office (DO/EO/US) received in the above-identified patent application, please amend the subject application as follows, in order to comply with the requirements of 37 CFR §§1.821-1.825:

In the Specification

Please substitute the paragraph on page 3, lines 4-13 with the following:

In order to obtain recombinant forms of human insulin analogues according to this invention, target genes were produced. This was accomplished by the "gap double-stranded DNA" method

described by Li Yiping *et al.* (1987, *Biotech. J.* 3:90) which permits site-directed mutations in the HI target gene. Primers specifically designed to give B12Thr, B16Ala and B26Ala were as follows;

For B12Thr (NHI-2): refer to Wang *et al.*, *supra*

For B16Ala (NHI-3): 5' TGA GGC TTT GNN STT GGT TTG CG 3' (SEQ ID No.1) in which N can be any nucleotide (G,A,T or C), and S is C or G.

For B26Ala (NHI-4): 5' GAA AGA GGTT TTC NNS ACT CCT AGG GC 3' (SEQ ID No.2) in which N and S are as defined above.

In the Sequence

Please replace original pages 1-3 (Sequence Listing) with new pages 1-2 attached hereto.

Remarks

By this amendment the nucleotide abbreviation "Y" has been changed to "S" to conform to the standard abbreviation for nucleotides "C or G." I hereby certify that no new material is being added by this submission.

The Commissioner is hereby authorized to charge any fees under 37 CFR 1.16 or 1.17 as required by this paper to Deposit Account No. 19-0065.

Respectfully submitted,

David Saliwanchik

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DRS/la
Attachment: New pages 1-2 (Sequence Listing) of the subject specification

Marked-up Version of Substitute Specification

Please substitute the paragraph on page 3, lines 4-13 with the following:

In order to obtain recombinant forms of human insulin analogues according to this invention, target genes were produced. This was accomplished by the "gap double-stranded DNA" method described by Li Yiping *et al.* ~~[6]~~ (1987, *Biotech. J.* 3:90) which permits site-directed mutations in the HI target gene. Primers specifically designed to give B12Thr, B16Ala and B26Ala were as follows;
For B12Thr (NHI-2): refer to Wang *et al.*, *supra*
For B16Ala (NHI-3): 5' TGA GGC TTT GNN ~~YTT~~ STT GGT TTG CG 3' (SEQ ID No.1) in which N can be any nucleotide (G,A,T or C), and ~~Y~~ S is C or G.
For B26Ala (NHI-4): 5' GAA AGA GGTT TTC ~~NNY~~ NNS ACT CCT AGG GC 3' (SEQ ID No.2) in which N and ~~Y~~ S are as defined above.

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Assistant Commissioner for Patents Washington, D.C. 20231 on July 8, 2002.

David Saliwanchik

David R. Saliwanchik, Patent Attorney

AMENDMENT UNDER 37 CFR §1.825(a) THROUGH (c)
Patent Application
Docket No. GJE-88
Serial No. 10/070,568

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	You-Min Feng, You-Shang Zhang
Serial No.	:	10/070,568
Filed	:	March 8, 2002
Conf. No.	:	7193
For	:	Monomeric Analogues of Human Insulin

Box PCT
Assistant Commissioner for Patents
Washington, D.C. 20231

AMENDMENT UNDER 37 CFR §1.825(a) THROUGH (c)

Sir:

In response to the Notification of Missing Requirements Under 35 U.S.C. 371 in the United States Designated/Elected Office (DO/EO/US) received in the above-identified patent application, please amend the subject application as follows, in order to comply with the requirements of 37 CFR §§1.821-1.825:

In the Specification

Please replace original pages 1-2 (Sequence Listing) with new pages 1-3 attached hereto.

H:\doc\pto\seq\GJE-88-amend.doc DNB/la

Remarks

This amendment is made to conform the application with the provisions of 37 CFR §§1.821 through 1.825. I hereby certify that no new material is being added by this submission.

The Commissioner is hereby authorized to charge any fees under 37 CFR 1.16 or 1.17 as required by this paper to Deposit Account No. 19-0065.

Respectfully submitted,

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DRS/la
Attachment: New pages 1-3 (Sequence Listing) of the subject specification

March 8, 2002

Patent Application
Docket No. GJE-88

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants : You-Min Fend and You-Shang Zhang
Docket No. : GJE-88
For : Monomeric Analogues Of Human Insulin

PRELIMINARY AMENDMENT

Please amend the above-identified patent application as follows:

In the Specification

Please add the following paragraph at page 1, above line 2:

This application is a National Stage Application of International Application Number PCT/GB00/03460, published, pursuant to PCT Article 21(2), in English.

After page 8: Please insert as new page 9 the attached Abstract of the Disclosure.

In the claims

The following amendments are made with respect to the claims in the international application PCT/GB00/03460 attached as Annexes to the International Preliminary Examination Report (IPER). Therefore, please replace existing page 8 of the international application with the amended claim sheet (replacement page 8) of the annex attached to the IPER, and make the following amendments to the pending claims so that they read as follows:

Claim 1 (amended):

An insulin analogue wherein the 16th or 26th amino acid of the B chain of human insulin (Tyr) is substituted by Ala, and which optionally also comprises a deletion at B1(Phe) and/or B30 (Thr).

Claim 2 (amended):

The insulin analogue, according to claim 1, wherein the 26th amino acid of the B chain of human insulin (Tyr) is substituted by Ala.

Claim 3 (amended):

The insulin analogue, according to claim 1, wherein the 26th amino acid is substituted by Ala, and which comprises a deletion at B30.

Claim 4 (amended):

The insulin analogue, according to claim 1, wherein the 16th amino acid of the B chain of human insulin (Tyr) is substituted by Ala.

Claim 5 (amended):

The insulin analogue, according to claim 1, wherein the 16th amino acid is substituted by Ala, and which comprises a deletion at B30.

Please add the following new claims:

6. A method for treating an individual having an insulin deficiency wherein said method comprises administering to the individual an insulin analogue wherein the 16th or 26th amino acid of the B chain of human insulin (Tyr) is substituted by Ala.

7. The method, according to claim 6, wherein said analogue has a deletion at B1 (Phe) or a deletion at B30 (Thr), or a deletion at both B1 and B30.

8. The method, according to claim 6, wherein, at the 26th amino acid, the analogue is substituted by Ala.

9. The method, according to claim 8, wherein said analogue has a deletion of B30.

10. The method, according to claim 6, wherein at the 16th amino acid, the analogue is substituted by Ala.

11. The method, according to claim 10, wherein said analogue has a deletion at B30.

12. A pharmaceutical composition comprising an insulin analogue wherein the 16th or 26th amino acid of the B chain of human insulin (Tyr) is substituted by Ala, wherein said composition further comprises a pharmaceutical carrier.

13. The pharmaceutical composition, according to claim 12, wherein said analogue has a deletion at B1 (Phe) or a deletion at B30 (Thr), or a deletion at both B1 and B30.

14. The pharmaceutical composition, according to claim 12, wherein at the 26th amino acid, the analogue is substituted by Ala.

15. The pharmaceutical composition, according to claim 14, wherein said analogue has a deletion of B30.

16. The pharmaceutical composition, according to claim 12, wherein at the 16th amino acid, the analogue is substituted by Ala.

17. The pharmaceutical composition, according to claim 16, wherein said analogue has a deletion at B30.

Remarks

Claims 1 through 5 have been amended and new claims 6-17 have been added. No new matter has been added by these amendments.

The Commissioner is hereby authorized to charge any fees under 37 CFR 1.16 or 1.17 as required by this paper to Deposit Account 19-0065.

Respectfully Submitted

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Marked-up Version of Amended Claims

Claim 1 (amended):

An insulin analogue wherein the 16th or 26th amino acid of the B chain of human insulin (Tyr) is substituted by Ala, and which optionally also comprises a deletion at B1(Phe) and/or B30 (Thr)[, for therapeutic use].

Claim 2 (amended):

The [An] insulin analogue, according to claim 1, wherein the 26th amino acid of the B chain of human insulin (Tyr) is substituted by Ala[(B26Ala)].

Claim 3 (amended):

The [An] insulin analogue, according to claim 1, wherein the 26th amino acid is substituted by Ala, and which comprises a deletion at B30[(des-B30, B26Ala)].

Claim 4 (amended):

The [An] insulin analogue, according to claim 1, wherein the 16th amino acid of the B chain of human insulin (Tyr) is substituted by Ala [(B16Ala)].

Claim 5 (amended):

The [An] insulin analogue, according to claim 1, wherein the 16th amino acid is substituted by Ala, and which comprises a deletion at B30 [(des-B30, B16Ala)].

Abstract of the Disclosure

Monomeric analogues of human insulin have a single substitution of the amino acid in 12th, 16th or the 26th position of the B chain of human insulin and may also have a terminal deletion in the B chain.

MONOMERIC ANALOGUES OF HUMAN INSULIN

Field of the Invention

This invention relates to novel monomeric analogues of human insulin (HI) obtainable by recombinant DNA technology.

Background of the Invention

Insulin is highly effective in treating insulin-dependent diabetes, and has been used clinically for nearly 80 years. With advances in DNA technology and the development of biotechnology industries, insulin extracted from animal pancreas is gradually being replaced by recombinant forms of human insulin, produced in microbial systems. This trend is encouraged by two observations; the number suffering from diabetes mellitus is on the increase globally and the clinical dose required to treat them is in milligram (mg) quantities.

Currently, the organisms employed for the commercial production of recombinant human insulin are *E. coli* and *S. cerevisiae*. The expression levels in *E. coli* are high but difficulties associated with downstream purification often lead to loss of yield. These difficulties are not encountered with *S. cerevisiae*, because the insulin produced is secreted into the culture medium, facilitating purification. However, the level of expression observed in this organism is low and difficult to increase.

Until recently, introduction of Lispro®, clinical preparations of human insulin contained polymeric forms of insulin which are slow-acting. Monomeric forms of insulin, as described in US-A-5618913, by contrast, are relatively fast-acting and mimic more closely the natural situation. They therefore demonstrate a great potential for clinical application. A commercial monomeric insulin, available as Lispro®, comprises inversion of amino acids 28 and 29 of the B chain of human insulin, and may be abbreviated as B28Lys,B29Pro.

Kristensen *et al*, J. Biol. Chem. 272(20):12978-83 (1997), discloses alanine substitution at various positions on the insulin molecule, including B12, B16 and B26. A single substitution with Ala affected the binding activity of the resultant insulin analogue in certain cases.

Wang *et al*, Biochem. Mol. Biol. Int. 39(6):1245-54 (1996), discloses B12Thr, i.e. an insulin analogue in which the 12th amino acid of the B-chain of human insulin (Val) is substituted by Thr. Again, an effect on binding activity was observed.

EP-A-0046979 discloses des-B30 derivatives of human insulin.

EP-A-0291863 discloses des-B1 derivatives of human insulin.

Summary of the Invention

According to the present invention, novel human insulin analogues are monomeric variants of B12Thr, B16Ala and B26Ala; the latter have not previously been recognised as monomeric. In addition to replacement of any or all of the 12th, 16th and 26th amino acids on the B-chain, such that the analogue is monomeric, the B-1 and/or B-30 terminal amino acids may be absent. The term "insulin analogue" as used herein means a compound having a molecular structure similar to that of human insulin, including disulphide bridges between A7Cys and B7Cys and between A20Cys and B19Cys, and an internal disulphide bridge between A6Cys and A11Cys, and having insulin activity.

Without wishing to be bound by theory, it appears that, in the primary structure of the insulin molecule, a number of the amino acids in the B-chain are responsible for the polymerisation of insulin in clinical preparations. These include those in positions B12, B16 and B26. In particular, the replacement of Val by Thr in position B12 or Tyr by Ala in position B16 or B26 significantly reduces the tendency of the insulin analogues to polymerise even at high concentrations (see Example 9). This enhanced tendency to exist as a monomeric structure is not affected by deletion of either one or both of the terminal amino acids of the B-chain.

Description of the Invention

The Scheme, below, shows the construction of the expression plasmids pNHI-2/AOX1, pNHI-3/AOX1, pNHI-4/AOX1and the engineering of recombinant cells YP99/NHI-2, YP99/NHI-3 and YP99/NHI-4. It sets out a representative procedure for the preparation of compounds of the invention, by analogy with the use of the human insulin target gene (HI) housed in the shuttle plasmid pHI/PGK. This shuttle vector is constructed from the plasmid pVT102-U (acquired from Canadian Research Institute) and subsequently multiplied by PCR (Maniatis *et al* (1989), Molecular Cloning A Laboratory Manual, 2nd ed. New York: Cold Spring Harbour Laboratory), to obtain multiple copies of human insulin target gene (HI) and flanking alpha mating factor leader (MFL) sequence. The target gene is then cloned into plasmid pPIC9 which is subsequently linearised with BglII prior to being employed to transform *P. pastoris* cell GS115 by the spheroplast method. Once plasmid pPIC9 containing the target gene is internalised, it integrates into the chromosomal DNA of the host cell [1]. Transformed cells bearing a high copy number of the HI gene are selected using the antibiotic G418 by the method described by Scover *et al* [2]. The presence of multiple copies of the HI are ascertained by the dot blotting method [3]. Cells bearing a high

copy number of the HI gene are utilised to generate the human insulin precursor by fermentation, and after purification converted to human insulin by tryptic transpeptidation.

In order to obtain recombinant forms of human insulin analogues according to this invention, target genes were produced. This was accomplished by the "gap double-stranded DNA" method described by Li Yiping *et al* [6] which permits site-directed mutations in the HI target gene. Primers specifically designed to give B12Thr, B16Ala and B26Ala were as follows;

For B12Thr (NHI-2): refer to Wang *et al, supra*

For B16Ala (NHI-3): 5' TGA GGC TTT GNN YTT GGT TTG CG 3' (SEQ ID No.1) in which N can be any nucleotide (G, A, T or C), and Y is C or G.

For B26Ala (NHI-4): 5' GAA AGA GGTT TTC NNY ACT CCT AGG GC 3' (SEQ ID No.2) in which N and Y are as defined above.

Novel human insulin analogues may be obtained by removing B30Thr and/or B1Phe, e.g. yielding a des-B1 and/or des-B30 analogue. Deletion may be achieved by known methodology. Rather than tryptic transpeptidation, to produce des-B30 human insulin, limited hydrolysis has been adopted, using trypsin in the preferred method, which further simplifies the process and increases the yield of insulin.

The methylotrophic yeast, *Pichia pastoris* is the preferred host for use in this invention for the preparation of insulin analogues because, as the Examples show, it has the advantages of high expression, simple processing, low production cost and high density culture. Furthermore it offers the advantages of a eukaryotic cell system; the correct folding and post-translational processing of secreted protein. These advantages greatly enhance the possibility of utilizing *P. pastoris* as the expression host in the scale-up of human insulin production. Its use in the expression of proteins of commercial importance has been documented elsewhere [3-5].

Human insulin analogues of the invention may be used in therapy. Their application and utility will be readily evident to those of ordinary skill in the art, e.g. in the treatment of diabetes mellitus.

Brief Description of the Drawings

Figure 1 shows the construction of pNHI-2/AOX1 plasmid of *Pichia pastoris*.

The following Examples illustrate the invention.

Example 1 Cloning of Mutated HI Gene

The plasmid pVT102-U from Canadian Biotechnology Research Institute was used to construct the plasmid pHI/PGK according to the standard method described

in Maniatis *et al* (1989). The construct pHI/PGK is a shuttle plasmid with phosphoglycerate kinase (PGK) promoter, followed by alpha mating factor leader sequence (MFL) to direct secretion of the product of the human insulin target gene (HI) flanked by a BamHI site at MFL 5' end and a HindIII site at HI 3' end. Using pHI/PGK as template, together with TCCGGATCCATGAGATTT (SEQ ID NO. 3) as the 5' primer and TGAATTCTTCTAGTTGCAGTAGTTT (SEQ ID NO. 4) as the 3' primer, DNA fragments containing MFL and HI with the BamHI site GGATCC at 5' end and the EcoR1 site GAATTC at the 3' end were obtained by PCR. To obtain DNA fragments containing MFL and the target gene NHI-2 (B12Thr), NHI-3 (B16Ala) and NHI-4 (B26Ala) the HI target gene in pHI/PGK plasmid was first mutated by site-directed mutagenesis then replicated by PCR. By inserting these fragments behind the AOX1 promoter of the plasmid pPIC9 (Invitrogen), expression plasmids pNHI-2/AOX1, pNHI-3/AOX1 and pNHI-4/AOX1 were obtained (see the Scheme and the accompany drawing; the latter shows the first plasmid, and the others may be prepared by the same procedure). The primers used to obtain the mutated genes in this invention have SEQ ID NOS. 1, 2 and 3.

Example 2 Construction and Screening of Expression Cell

The expression plasmids were linearised by BglII and used to transform *P. pastoris* cell GS115 (Invitrogen) using the spheroplast method. The linearised plasmids, once internalized, integrate into the chromosomal DNA of the host cell [1]. The recombinant cells, designated YP99/NHI-2, YP99/NHI-3 and YP99/HNI-4 with high copy number of the target gene, were selected by antibiotic G418 [2] and identified by the dot blotting method [3].

Example 3 Preparation of Precursors of HI analogues

High density fermentation was carried out in a 15 litre fermenter [7]. The following salt solutions were used in the fermentation: BSM - H_3PO_4 26.7 ml/l, $CaSO_4.H_2O$ 0.93 g/l, K_2SO_4 18.2 g/l, $MgSO_4.7H_2O$ 14.9 g/l, KOH 4.13 g/l; PTM1 - $CuSO_4.5H_2O$ 6 g/l, KI 0.08 g/l, $MnSO_4H_2O$ 3.0 g/l, $NaMoO_4.H_2O$ 0.2 g/l, H_3BO_3 0.02 g/l, $CoCl_26H_2O$ 0.5 g/l, $ZnSO_4$ 20.0 g/l, H_2SO_4 5 ml/l, $FeSO_4.7H_2O$ 65.0 g/l.

Fermentation medium containing 6 L of salt solution BSM and 300 ml of glycerol is sterilised in the fermenter. Its pH is adjusted to 5.5 with 50% ammonium hydroxide. A 5 ml aliquot of salt solution PTM1 containing 1 mg of biotin is added per 1 litre of culture medium. The expression cell is inoculated to 50 ml YPG and grown in a shake flask at 30°C for 24 hr. The broth is added to 600 ml of YPG, shaken in 3 flasks for 24 hr, added to the culture medium and fermented for 24 hr to deplete

glycerol. Methanol solution containing PTM1 (5 ml/l) and biotin (1 mg/l) is added to induce the expression. The inductive fermentation is continued for 84 hr by feeding the above methanol solution. During the fermentation, the pH is maintained at 5.5 by adding 50% ammonium hydroxide. The expression level is measured by radioimmunoassay, SDS-polyacrylamide gel electrophoresis [8] and HPLC.

Example 4 Separation and Purification of the Precursors

The fermentation broth is centrifuged to remove the cell bodies. The supernatant is applied to a C8 column and purified by HPLC. After a single step of purification, a product can be obtained that is homogeneous in native polyacrylamide gel electrophoresis.

Example 5 Transpeptidation of the Precursors

Purified precursors of HI analogues from Example 4 are dissolved in DMSO/1,4-butanediol/H_2O (15:70:15, v/v) to a concentration of 30 mg/ml. Thr(Bu^t)-OBu^t is added in excess, and the pH is adjusted to 6.5 by ammonium hydroxide. TPCK-trypsin is added (substrate:enzyme = 5:1) and the reaction mixture is incubated at 25°C for 6 hr. The reaction is stopped by acidification. The product is precipitated using acetone, and purified by HPLC using C8 column.

Example 6 Preparation of des-B30 analogues

Purified precursors of HI analogues are dissolved in pH 8, 0.1M ammonium bicarbonate to a concentration of 10 mg/ml. TPCK-trypsin is added (substrate:enzyme = 200:1) and the reaction mixture is incubated at 25°C overnight. The product is analysed by native polyacrylamide gel electrophoresis

Example 7 Preparation of des-B1 analogues

HI analogues are reacted with phenylisocyanate in a molar ratio of 1:2, prior to treatment with trifluoroacetic acid as described by Bradenburg & Hoppe-Seyler, Physiol. Chem. 350:471. The products of this reaction are separated and analysed by electrophoresis and found to be almost exclusively des-B1 forms of insulin analogues.

Example 8 Preparation of des-B1, des-B30 analogues

Prepared by processing precursors of HI analogues as described in Example 6 followed sequentially by that described in Example 7.

Example 9 Determination of structural forms

The structural form of the recombinant human insulin analogues prior to deletion of the one or both terminal amino acids of the B-chain is determined electrophoretically. A preparation of each analogue is passed through Superdex G-75 column (HR 10/30). HI and [B28Lys, B29Pro] insulin (Lispro) are used as negative

and positive controls respectively. Phosphate buffered saline pH 7.4 is used as an elution buffer and the flow rate fixed at 0.4 ml/min. The concentration of the sample preparation is 1.2 mg/ml. The retention times and the peak profiles of human insulin analogues are shown in the following Table.

Sample	Retention Time, min	Peak profile
HI	36.4	Unsymmetrical
[B28Lys, B29Pro]HI	39.4	Symmetrical
[B12Thr]HI	39.4	Symmetrical
[B16Ala]HI	38.3	Symmetrical
[B26Ala]HI	38.9	Symmetrical

These results demonstrate that HI analogues B12Thr, B16Ala and B26Ala are all monomeric in form. They have a similar retention time and peak profile as the known positive control [B28Lys, B29Pro] human insulin.

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Scheme

pVT102-U (Canadian Biotech Res Inst.)

↓ Removal of ADHp and replacement by PGKp together with the addition of α-MFL sequence & HI precursor gene

pHI/PGK (shuttle plasmid) with HI precursor gene & α-MFL sequence

↓ Site-directed mutagenesis using primers of SEQ1, SEQ2 & SEQ3

pNHI-2, pNH-3, or pNHI-4/PGK with Novel HI precursor genes & α-MFL sequence

↓ PCR; multiplication of novel target gene

Production of Multiple copies of NHI-2, NHI-3 or NHI-4 precursor genes & α-MLF

↓ Novel precursor genes & α-MFL fragment tailored to lie between BamHI and EcoR1 site. These fragments are inserted into the pPIC9 plasmid just after the AOX1 promoter

Expression plasmid; pNHI-2/AOX1 or pNHI-3/AOX1 or pNHI-4/AOX1

↓ Expression plasmid linearised with Bg III & used to transform GS 115 cells

P. pastoris (GS 115 cells) transformation

↓ Screen transformed cells for the production novel HI precursors; check gene sequence then select for high yielding cells with G418

Transformants YP99/NHI-2, YP99/HNI-3,& YP99/NHI-4 cells

↓ Grow cells in BMS salt solution, induce with methanol, purify novel HI precursor, covert to human insulin analogues, then modify through terminal deletion(s) in the B-chain

CLAIMS

1. An insulin analogue wherein the 16th or 26th amino acid of the B chain of human insulin (Tyr) is substituted by Ala, and which optionally also comprises a deletion at B1(Phe) and/or B30 (Thr), for therapeutic use.

2. An insulin analogue wherein the 26th amino acid of the B chain of human insulin (Tyr) is substituted by Ala (B26Ala).

3. An insulin analogue according to claim 1, wherein the 26th amino acid is substituted by Ala, and which comprises a deletion at B30 (des-B30, B26Ala).

4. An insulin analogue wherein the 16th amino acid of the B chain of human insulin (Tyr) is substituted by Ala (B16Ala).

5. An insulin analogue according to claim 1, wherein the 16th amino acid is substituted by Ala, and which comprises a deletion at B30 (des-B30, B16Ala).

ART 34 AMDT

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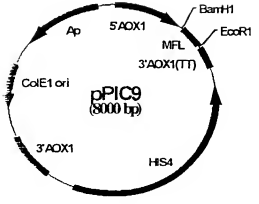
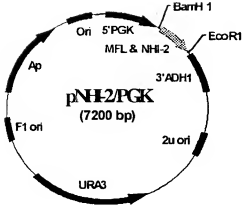
(54) Title: MONOMERIC ANALOGUES OF HUMAN INSULIN

(57) Abstract: Monomeric analogues of human insulin have a single substitution of the amino acid in 12th, 16th or the 26th position of the B chain of human insulin and may also have a terminal deletion in the B chain.





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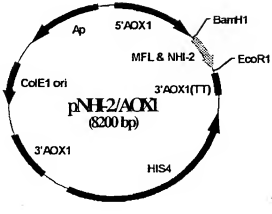

Figure 1

USA

DECLARATION AND POWER OF ATTORNEY

As a below-named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name; I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of subject matter which is claimed and for which a patent is sought on an invention entitled
MONOMERIC ANALOGUES OF HUMAN INSULIN

the specification of which ☐ is attached hereto or

☒ was filed on 08 SEP 2000 as United States Application Number or PCT International Application Number PCT/GB00/03460 and was amended on 19 NOV 2001 (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56. I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for a patent or inventor's certificate, or PCT international application having a filing date before that of the application on which priority is claimed:

Prior Foreign Application Number(s)	Country	Foreign Filing Date	Priority Not Claimed	Certified Copy Attached? YES	NO
99116851.8	CN	08 SEP 1999	☐	☐	☐
			☐	☐	☐
			☐	☐	☐

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C 1001 and that such willful false statements may jeopardise the validity of the application or any patent issued thereon.

1-00

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Country of Citizenship: P.R. China Date of signature: 8/2/02

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